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Screening for Abnormal Cholesterol Biosynthesis in the Smith-Lemli-Opitz Syndrome: Rapid Determination of Plasma 7-Dehydrocholesterol by Ultraviolet Spectrometry

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The Smith-Lemli-Opitz syndrome (SLOS) is a common condition caused by deficiency of 7-dehydrocholesterol Δ^7-reductase. The syndrome can usually be diagnosed by demonstrating markedly increased plasma concentrations of the cholesterol precursor, 7-dehydrocholesterol. We describe a simple and rapid method for detection of plasma 7-dehydrocholesterol by use of ultraviolet (UV) spectrometry. Lipids were extracted from plasma by addition of ethanol and n-hexane, and the n-hexane phase was directly subjected to spectrometry. The absorption maxima characteristics of 7-dehydrocholesterol (λ_{max} 271, 282, and 294 nm) were observed in patients' plasma but not in controls. For quantitative measurements, absorbance at 282 nm was used. Since this absorbance is the sum of the absorbance derived from 7-dehydrocholesterol and background absorbance, the concentrations of 7-dehydrocholesterol in various plasma samples were quantified by subtracting estimated background absorbance at 282 nm from observed absorbance at 282 nm. The results correlated well with total (free plus esterified) 7-dehydrocholesterol concentrations measured by gas-liquid chromatographic method. The UV spectrometric assay was sensitive enough to detect increased 7-dehydrocholesterol in cultured skin fibroblasts from patients grown in delipidated medium. The present method will make it possible to screen plasma or fibroblasts to detect the syndrome rapidly in general clinical laboratories. Am. J. Med. Genet. 68:288–293, 1997. © 1997 Wiley-Liss, Inc.

KEY WORDS: Smith-Lemli-Opitz syndrome; screening; plasma; skin fibroblast; 7-dehydrocholesterol; ultraviolet spectrometry

INTRODUCTION

The Smith-Lemli-Opitz syndrome (SLOS) [Smith et al., 1964] is an autosomal recessive disorder caused by deficient activity of 7-dehydrocholesterol Δ^7-reductase [Honda et al., 1995; Shefer et al., 1995] that results in abnormally low tissue cholesterol and accumulation of precursor, 7-dehydrocholesterol and its isomer, 8-dehydrocholesterol [Batta et al., 1995; Tint et al., 1995]. Patients are characterized clinically by mental retardation, failure to thrive, and multiple organ anomalies [Smith et al., 1964; Chasalow et al., 1985; Curry et al., 1987; Gorlin et al., 1990; Pober et al., 1990]. The prevalence is estimated to be about 1 in 20,000 births with a carrier frequency between 1% and 2% in North American Caucasian populations. However, it is suspected that when newborn screening is performed, this syndrome will be found to be even more common [Opitz, 1994].

The clinical diagnosis of the syndrome cannot always be made with certainty because of the remarkable variability of clinical expression [Opitz, 1994]. However, it can usually be diagnosed by demonstrating markedly increased plasma concentrations of 7-dehydrocholesterol [Irons et al., 1993; Tint et al., 1994] by gas-liquid chromatography (GLC) [Batta et al., 1995; Tint et al., 1994] or gas chromatography-mass spectrometry (GC-MS) [Kelley, 1995; Tint et al., 1995]. These methods are sensitive but not convenient to determine in general

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clinical laboratories or to screen a large number of samples because special apparatus is required and one must hydrolyze esterified 7-dehydrocholesterol in order to measure total 7-dehydrocholesterol concentration.

7-Dehydrocholesterol (Fig. 1) shows characteristic ultraviolet (UV) absorption maxima (λ_{max}) at 271, 282, and 294 nm [Nes, 1985]. Since substantial amounts of 7-dehydrocholesterol are present in the plasma from SLOS patients, Batta et al. [1994] suggested the possibility that UV spectrometric assay could be useful for rapid screening of the syndrome. UV spectrometry is more extensively used in clinical laboratories and the hydrolysis step is not required for determination of total 7-dehydrocholesterol concentrations when measured by UV absorbance. In this paper, we describe a simple, rapid, and accurate UV spectrometric method for detection and quantitation of 7-dehydrocholesterol in plasma and cultured skin fibroblasts from SLOS patients and compare the results with those obtained by GLC.

MATERIALS AND METHODS

Patients

We studied 23 patients (10 females and 13 males, age 0–26 years). Except for patient 29, clinical and biochemical (plasma sterol concentrations) presentations of the patients were consistent with the SLOS. Patient 29 is a 26-year-old man originally described by Johnson [1975] and is biochemically atypical because he has normal plasma cholesterol level (154 mg/dl) and a 7-dehydrocholesterol concentration of only 0.15 mg/dl. However, he manifested the syndrome and showed deficient activity of 7-dehydrocholesterol Δ^7-reductase in cultured skin fibroblasts [Honda et al., 1995].

Chemicals

Reference standard of 7-dehydrocholesterol was purchased from Steraloids, Inc. (Wilton, NH). [1,2-^{3}H] 7-dehydrocholesterol was synthesized as described previously [Shefer et al., 1995]. Spectral quality n-hexane was purchased from Fisher Scientific Co. (Pittsburgh, PA).

Blood Sample Collection

Blood samples from the patients and 30 apparently healthy adult people were collected into tubes with or without EDTA. After centrifugation, plasma or serum was stored at −20°C until analyzed.

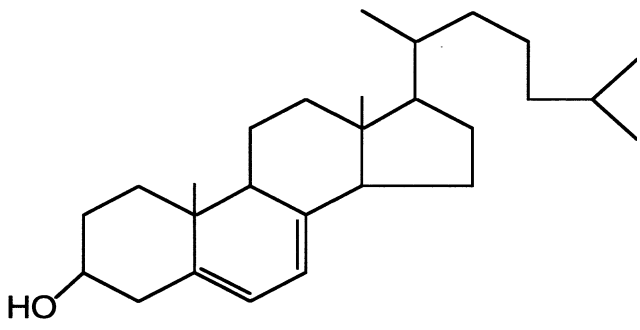

Fig. 1. Structure of 7-dehydrocholesterol.

Fibroblast Culture

Skin fibroblasts were grown and maintained as monolayers in Dulbecco's Modified Eagle Medium (D-MEM; Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS). When the cells were nearly confluent, the original growth medium was removed, the attached cells washed twice with phosphate-buffered saline (PBS), and the medium replaced with fresh D-MEM containing 2.5 mg/ml of delipidized protein from FBS [Capriotti and Laposata, 1987]. Cell growth was maintained for 2 weeks at 37°C in a humidified incubator containing 5% CO_2 and 95% air, and the medium was replaced with fresh delipidated medium twice per week.

UV Spectrometric Analysis

A 200-µl aliquot of plasma or serum was transferred to a 12 × 75 mm glass tube. The tube was vortexed for 10 seconds after addition of 200 µl of ethanol. Then 1 ml of n-hexane was added and the mixture vortexed for another 20 seconds. After centrifugation at 400 g for 1 min, the clear n-hexane layer was collected in a 1-mL quartz cuvet and was used for UV measurement on a Gilford system 2600 spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, OH). The samples were scanned using a n-hexane blank in the reference beam, and the absorption maxima at 271, 282 and 294 nm were used for detection of 7-dehydrocholesterol in plasma samples while the absorption maxima at 234 and 282 nm were used for quantitative analysis.

Cultured skin fibroblasts grown in 25-cm^2 tissue culture flasks (approximately 1×10^6 cells/flask) were harvested by use of cell scrapers and washed twice with PBS. Cells were extracted with 200 µl of ethanol in an ultrasonic bath for 15 min. After addition of 200 µl of water and 1 ml of n-hexane, lipids were extracted as described for plasma and subjected to UV measurement.

GLC Analysis

Plasma 7-dehydrocholesterol concentrations were determined by capillary-column GLC as described previously [Batta et al., 1995; Tint et al., 1995].

Statistics

Data are reported as the mean ± S.D. The statistical significance of differences between the results in the different groups was evaluated with the Mann-Whitney two-tailed test. Linear regression analysis or two-dimensional polynomial regression analysis was used to verify the correlation.

RESULTS

Recovery of 7-Dehydrocholesterol

For UV spectrometric measurement, plasma-free plus esterified 7-dehydrocholesterol were extracted once by addition of 6 volumes of n-hexane-ethanol (5:1, v/v), while for GLC measurement, plasma-free 7-dehydrocholesterol obtained by hydrolysis with ethanolic NaOH was extracted three times with n-hexane [Batta et al., 1995; Tint et al., 1995]. The recovery of 7-dehydrocholesterol by these extraction procedures was de-

termined by addition of ^{3}H-labeled 7-dehydrocholesterol to plasma and was found to be 91.1 ± 3.0% (n = 7) and 96.4 ± 2.8% (n = 7), respectively.

Detection of SLO Patients

Figure 2 shows UV spectra of standard 7-dehydrocholesterol (Fig. 2a) and plasma extracts from a typical patient (patient 58) and a biochemically atypical patient (patient 29; Fig. 2b), and from controls (Fig. 2c). Plasma from all patients showed UV absorption maxima (λ_{max}) at 271, 282, and 294 nm characteristic of 7-dehydrocholesterol. In contrast, control plasma did not show these characteristic absorption maxima. However, plasma from patients and controls showed a wide range of magnitude of background absorbance between 220 and 320 nm and an absorption maximum of the background absorbance was around 234 nm.

Absorbance at 282 nm and the ratio of absorbances at 282 nm to 234 nm (absorbance at 282/234 nm) in individual plasma samples are shown in Figure 3. As seen from Figure 3a, absorbance at 282 nm was significantly higher in patients than in controls (0.75 ± 0.34 vs. 0.18 ± 0.17, $P < 0.0001$); however, overlaps were observed. We also tried to use absorbance at 271 or 294 nm instead of 282 nm, but separation was essentially the same as that for 282 nm. In contrast, when we calculated the absorbance at 282/234 nm, not only was the 282/234 ratio significantly higher in plasma from the patients than in controls (1.48 ± 0.83 vs. 0.10 ± 0.03, $P < 0.0001$), plasma from all patients except for patient 29 was completely discriminated from controls (Figure 3b).

Quantitative Analysis of 7-Dehydrocholesterol

The absorbance at 282 nm (A_{282}) is the sum of the absorbance derived from 7-dehydrocholesterol (B) and the background absorbance (C). $A_{282} = B + C$. The absorbance at 234 nm (A_{234}) is sum of the absorbance due to 7-dehydrocholesterol (D) and background absorbance (E). $A_{234} = D + E$.

In an experiment using standard 7-dehydrocholesterol (Fig. 2a), absorbance at 234 nm due to 7-dehydrocholesterol (D) was estimated as about 14.7% of the absorbance at 282 nm (B). D = 0.147B. Strong correlation between absorbances at 234 nm and 282 nm in control plasma was observed, and the absorbance at 282 nm (C) could be estimated from the absorbance at 234 nm (E; Fig. 4). $C = 0.024E^2 + 0.046E + 0.01$.

By using above four equations, it was possible to calculate the absorbance (B) due to 7-dehydrocholesterol as follows:

$$B^2 - (13.63A_{234} - 1917.37)B + A_{234}(46.33A_{234} + 88.80) - 1930.50A_{282} + 19.31 = 0.$$

Therefore,

$$B = \frac{13.63A_{234} - 1917.37 + \sqrt{(13.63A_{234} - 1917.37)^2 - 4\left[A_{234}(46.33A_{234} + 88.80) - 1930.50A_{282} + 19.31\right]}}{2}$$

Calibration curve was obtained for 7-dehydrocholesterol after adding known amounts of 7-dehydrocholesterol to 6 control plasma samples, and a good correlation was observed between the absorbance at 282 nm due to 7-dehydrocholesterol (calculated from the above final equation) and amounts of 7-dehydrocholesterol added in the range 1–38 mg/dl (Fig. 5).

Table I compares the plasma total (free plus esterified) 7-dehydrocholesterol concentrations quantified by UV spectrometric and GLC methods. The coefficient of variation of the spectrometric method, as calculated from 7 replicate determinations of plasma from a typical patient (patient 59), was 1.3%. The amounts of 7-dehydrocholesterol in the patients determined by the present spectrometric method (y) correlated well (R = 0.950, $P < 0.0001$, n = 23) with total 7-dehydrocholesterol concentrations quantified by the GLC method (x),

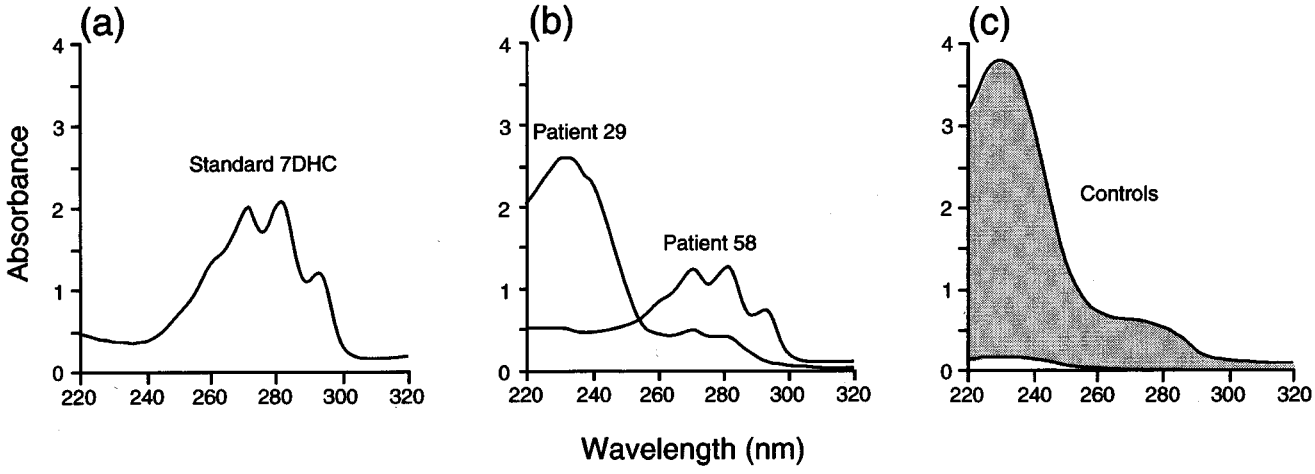

Fig. 2. **a**: UV spectra of standard 7-dehydrocholesterol (38 mg/dl). **b**: UV spectra of plasma extracts from a typical patient (patient 58) and a biochemically atypical patient (patient 29). **c**: A range of the magnitude of UV spectra of plasma extracts from 30 controls (shaded area).

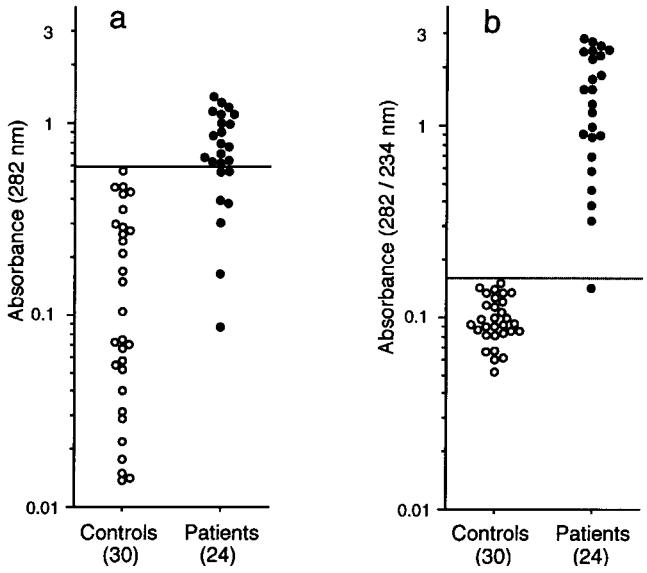

Fig. 3. Absorbance at 282 nm (**a**) and the ratio of absorbances at 282 nm to 234 nm (**b**) in plasma from Smith-Lemli-Opitz patients (closed circles) and controls (open circles). Horizontal bars indicate the upper limits of control ranges; number of subjects studied is given in parentheses.

and the linear regression equation was y = 0.931x + 0.410. In patient 29, 7-dehydrocholesterol concentration, which was determined to be 0.15 mg/dl by GLC method, was too low to quantify by the present spectrometric assay, and thus, the lower limit of detection of 7-dehydrocholesterol by this method is approximately 0.2–0.5 mg/dl.

Application to Cultured Skin Fibroblasts

The lipid extracts from cultured skin fibroblasts grown in delipidated medium for 2 weeks were subjected to UV spectrometry. The absorption maxima characteristics of 7-dehydrocholesterol were observed

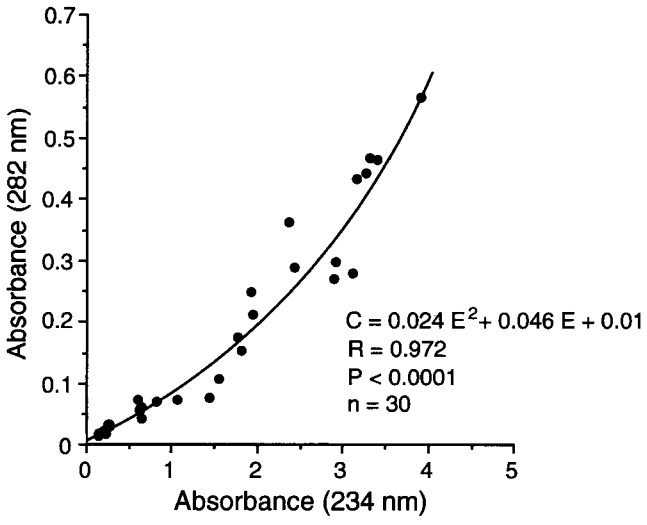

Fig. 4. Correlation between the absorbances at 234 nm and 282 nm in control plasma. In controls, the background absorbance at 282 nm (C) could be estimated from the absorbance at 234 nm (E) according to the equation: $C = 0.024E^2 + 0.046E + 0.01$.

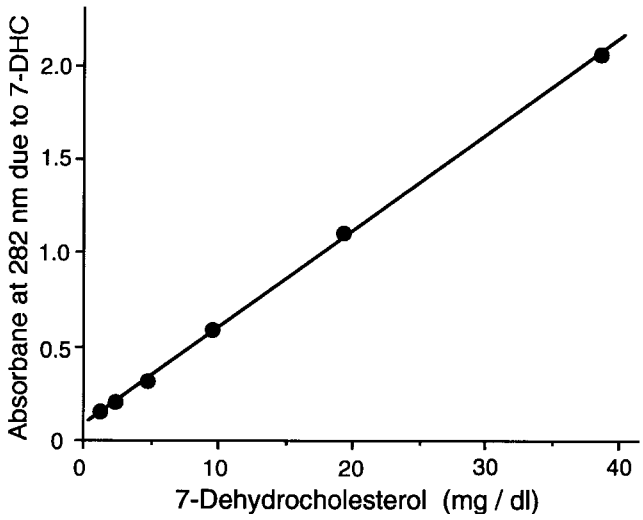

Fig. 5. Calibration curve for 7-dehydrocholesterol. Varying amounts of 7-dehydrocholesterol were added to control plasma. Absorbance at 282 nm due to 7-dehydrocholesterol was calculated by the equation in the text.

in all fibroblasts from patients irrespective of plasma 7-dehydrocholesterol concentrations, but not in control fibroblasts. Table II shows the absorbance at 282/234 nm, absorbance at 282 nm due to 7-dehydrocholesterol and 7-dehydrocholesterol concentrations in cell extracts from four patients (patients 9, 29, 36 and 38) who

TABLE I. Comparison of UV Spectrometry and GLC Methods for Measuring Total 7-Dehydrocholesterol Concentrations in Plasma From Smith-Lemli-Opitz Patients

	Method		
Patient	UV (mg/dl)	GLC (mg/dl)	Difference[a] (%)
1	19.2	18.8	2.1
2	18.6	20.9	−11.2
4	8.2	7.3	12.5
6	11.7	13.5	−13.4
9	3.9	4.4	−10.8
12	3.6	4.5	−20.3
13	9.2	11.7	−21.8
18	9.8	12.5	−21.5
24	19.2	24.0	−19.9
26	18.6	22.0	−15.4
26T[b]	10.8	13.7	−21.2
29	NQ[c]	0.15	—
33	13.7	12.8	7.3
36	0.5	0.44	13.6
38	1.0	1.7	−39.3
40	23.6	21.7	8.6
43	14.7	13.6	7.8
44	8.6	9.5	−9.1
49	8.9	8.0	11.2
50	16.5	14.5	13.8
53	16.9	13.1	28.7
58	22.1	21.7	1.9
59	10.2	12.0	−14.9
63	12.9	11.0	17.5

[a] Calculated as [UV data-GLC data] divided by GLC data × 100%.
[b] During treatment with high cholesterol diet.
[c] Not quantifiable.

TABLE II. Determination of 7-Dehydrocholesterol (7-DHC) in Cultured Skin Fibroblasts From Smith-Lemli-Opitz Patients and Controls*

Subject	Absorbance (282/234 nm)[b]	Absorbance at 282 nm due to 7-DHC[c]	7-DHC concentration[a] (μg/flask[d])
Patients			
9	0.38	0.16	2.9
29	0.37	0.33	9.2
36	0.29	0.14	1.9
38	0.57	0.27	6.8
Mean ± S.D.	0.40 ± 0.12[e]	0.22 ± 0.09	5.2 ± 3.4[e]
Controls			
Mean ± S.D. (n = 4)	0.051 ± 0.005	<0.00	NQ[f]
Range	0.046–0.059		

* Fibroblasts from patients and controls were exposed to delipidated media for 2 weeks.
[a] Concentrations of 7-dehydrocholesterol were calculated by substituting the absorbance at 282 nm (column 3) in Figure 5.
[b] Ratio of absorbance at 282 nm to absorbance at 234 nm.
[c] Absorbance at 282 nm derived from 7-dehydrocholesterol obtained by subtracting background absorbance at 282 nm from the observed absorbance at 282 nm (calculated from the equation in the text).
[d] Confluent cells in a 25-cm^2 tissue culture flask (approximately 1×10^6 cells/flask).
[e] $P < 0.05$, significantly different from controls.
[f] Not quantifiable.

exhibited the lowest plasma levels of 7-dehydrocholesterol. In patients, absorbance at 282/234 nm was 8 times higher ($P < 0.05$) than that in controls and all 4 patients were completely discriminated from controls. When we calculated the concentrations of 7-dehydrocholesterol in cells by the method described for plasma, accumulated 7-dehydrocholesterol was observed ($P < 0.05$) in all 4 patients but not in controls.

DISCUSSION

Although determination of plasma 7-dehydrocholesterol is highly effective for biochemical diagnosis of the SLOS [Irons et al., 1993; Tint et al., 1994], the measurement has been performed in only specialized laboratories because previous methods are not feasible for routine clinical test. The UV spectrometric method presented here is very simple and rapid and can be easily adapted in general clinical laboratories. Furthermore, this method has the advantage over the existing methods that one can detect total 7-dehydrocholesterol (including esters) in the plasma or tissues without prior hydrolysis since both free and esterified 7-dehydrocholesterol show identical UV absorbance and extinction coefficients. This was confirmed in a set of experiments where sterols were extracted from aliquots of plasma prior to or after hydrolysis. The concentrations of 7-dehydrocholesterol were found to be identical (within 10%) in both cases.

This spectrometric method takes advantage of the characteristic strong UV absorption maxima (λ_{max}) at 271, 282 and 294 nm due to the homoannular $\Delta^{5,7}$-diene system in 7-dehydrocholesterol. Other sterols present in the SLOS, viz. cholesterol and 8-dehydrocholesterol which lack a conjugated diene system, do not absorb in this UV region and are not detected. The method is not as sensitive as the GLC [Tint et al., 1994; Batta et al., 1995], GC-MS [Kelley 1995; Tint et al., 1995] and high-performance liquid chromatography (HPLC) [Axelson, 1991] methods. However, it appears to be sensitive enough to detect plasma 7-dehydrocholesterol to establish the diagnosis of the syndrome. Although the characteristic absorption maxima at 271, 282 and 294 nm were not prominent in the atypical patient 29, 7-dehydrocholesterol concentration of this patient (0.15 mg/dl) is within the range found in non-SLOS subjects [Axelson, 1991] and the level is considerably below that reported for most individuals with the syndrome. We have measured 7-dehydrocholesterol concentrations in plasma from 77 SLOS patients by GLC method, and found that only patients 29 and 36 had less than 1 mg/dl of 7-dehydrocholesterol and only 4 patients including patients 29 and 36 had less than 5 mg/dl of 7-dehydrocholesterol (unpublished results).

The background absorbance at 282 nm appears to arise from substances which have an absorption maximum around 234 nm since strong positive correlation between absorbances at 234 nm and 282 nm in control plasma was observed (Fig. 4), and we could eliminate the effect of background absorbance at 282 nm by calculating the ratio 282/234 nm. Therefore, the measurement of absorbance at 282/234 nm was found to be more reliable for detection of the syndrome than absorbance at 282 nm alone (Fig. 3b vs. Fig. 3a). Absorbance at 234 nm seems to derive from oxidized lipids (e.g., cholesterol oxidation products [Sevanian et al., 1995] or oxidized polyunsaturated fatty acids [Puhl et al., 1994], etc.) because plasma frozen and thawed several times was found to show higher absorbance at 234 nm while lipoprotein deficient plasma prepared from one of the control subjects exhibited very low absorbance (<10%) at 234 nm compared with the original plasma.

Not only detection but also quantitative analysis of 7-dehydrocholesterol was possible by using the present UV spectrometric assay (Table I). Although the limit of quantification was between 0.2 and 0.5 mg/dl, which is higher than those of the previous methods, except for atypical cases, this sensitivity was enough to quantify 7-dehydrocholesterol in plasma from SLOS patients. It may be mentioned here that even though the proteins and bilirubin present in the plasma do not interfere with this assay, drugs which, or the metabolite of which, absorb at either 282 or 234 nm, when adminis-

tered to patients, may interfere with the quantitation of 7-dehydrocholesterol in the plasma.

Cultured skin fibroblasts are an excellent model for in vitro study of cholesterol biosynthetic pathway, and the cells from SLOS patients manifest the deficiency of 7-dehydrocholesterol Δ^7-reductase [Honda et al., 1995]. Although 7-dehydrocholesterol concentrations in fibroblasts from patients were not elevated proportionately as much as in plasma in the presence of 10% FBS, the concentrations in patients including the atypical patient 29 increased markedly under cholesterol-depleted conditions [Honda et al., 1997]. The measurement of 7-dehydrocholesterol in fibroblasts after exposing to delipidated medium is a more sensitive procedure for screening of the SLOS than plasma sterol analysis, and the UV spectrometric assay could be easily adapted to detect accumulated 7-dehydrocholesterol in cultured skin fibroblasts from patients.

In summary, we have established a simple UV spectrometric method for detection and quantification of 7-dehydrocholesterol in plasma and cultured skin fibroblasts from patients with the SLOS. The method will make it possible to screen for the syndrome rapidly in general clinical laboratories.

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